Final Technical Report

DARPA-AFOSR Award ID: F49620-03-0320

MULTIFUNCTIONAL MAGNETIC NANOPARTICLE PROBES FOR DEEP-TISSUE IMAGING

May 1, 2003 – October 31, 2004

Principal Investigator

Gang Bao, Professor
Department of Biomedical Engineering
Georgia Institute of Technology and Emory University
Atlanta, GA 30332

June 8, 2005

1. OBLECTIVES

The goal of the DARPA-AFOSF project is to develop multifunctional magnetic nanoparticle probes for deep-tissue imaging using MRI. The specific objectives of the Phase I project include: (1) to functionalize iron-oxide magnetic nanoparticles for bioconjugation of oligonucleotides and peptides; (2) proof-of-concept demonstration of the signal transduction mechanism based on nanoprobe clustering on mRNA target; (3) to develop peptide-based delivery of magnetic nanprobes into living cells with high delivery efficiency; (4) to perform preliminary MRI studies of detection sensitivity and signal-to-noise ratio in solution and in cells. This innovative molecular imaging approach integrates *in vivo* delivery, targeting/sensing and signal transduction; it has the potential to revolutionize medical imaging, diagnosis, and therapeutics with many DoD applications.

2. STATUS OF EFFORT

During the Phase I studies of the project, we have functionalized iron-oxide magnetic nanoparticle surface with a phospholipid-PEG coating. The resulting nanoparticles are water-soluble and suitable for bioconjugation of oligonucleotides and peptides. We have conjugated linear and hairpin oligonucleotide probes to magnetic nanoparticles and confirmed hybridization-induced clustering. We have developed a peptide-based delivery method for nanoparticle probes. This method has very high delivery efficiency and fast kinetics compared with other transfection-based methods. We have performed spectroscopic and MR imaging studies of the effect of magnetic nanoparticle probe coating and correlated the experimental measurements with theoretical prediction. We have also developed a saturation by off-resonance irradiation method which gives better signal-to-noise ratios. Taken together, these studies have greatly advanced the development of magnetic nanoparticle probes as an MRI contrast agent for deep tissue imaging.

3. ACCOMPLISHMENTS/NEW FINDINGS

A. Functionalization of Magnetic Nanoparticles

Magnetic iron oxide nanoparticles (MIONs) with chemical composition Fe_3O_4 and average diameter of 6 nm were provided by Dr. Charles O'Connor at the University of New Orleans. The first major challenge to overcome was to make MIONs water-soluble and create a stable suspension of MIONs that are biocompatible and surface-functionalized. An approach employed by some other investigators in the field is to use the polysaccharide dextran. A distinct disadvantage to this approach is the large thickness (~15 nm) of the coating. In order to minimize steric hindrance in hybridization between two probes, it is crucial to minimize the size of the coating. Therefore, we have focused our initial efforts on developing alternative coatings. In our approach, superparamagnetic iron oxide nanoparticles (MIONs) are encapsulated in a PEG-modified phospholipid micelle structure to generate micelle-encapsulated MIONs (mMIONs). This approach exploits the hydrophobic nature of the nanoparticle surface by utilizing an amphiphilic PEG-phospholipid, whose hydrophobic portion interacts with the nanoparticle surface to create micelles, resulting in a self-assembled monolayer

coating. The PEG portion of the coating confers solubility and biocompatibility while the use of modified PEG allows for bioconjugation of proteins, multiple ligands, and nucleic acid probes.

B. Size of Functionalized Nanoparticles

Using dynamic light scattering (DLS) measurements, the average hydrodynamic radius of the mMIONs was 7.34 nm ± 0.71 nm (93.9% of mass). The size range of the coated MIONs obtained with DLS is similar to that reported by for micelle-encapsulated quantum dots. The DLS results also indicated a very narrow range of polydispersity in mMIONs, which may provide a crucial advantage for molecular imaging applications based on clustering of magnetic nanoparticles. Electron microscopy was also performed to verify the presence of coating and to determine the coating thickness. Negative staining allowed a contrast between the background and the dark iron core, revealing an unstained micelle coating around the MIONs. The size of mMIONs as determined by the TEM image is between 12 to 14 nm, similar to the results obtained by DLS. This indicates that the coating thickness is around 3 nm.

C. Cellular Delivery of Nanoparticle Probes and Their Functionality

To demonstrate the functionality of magnetic nanoparticle probes, we used Tat peptide for nanoparticle delivery into living cells. Specifically, fluorescent dye molecules and delivery peptides were simultaneously attached to the surface of the mMIONs through a reactive amine group. As a member of the cell penetrating peptide (CPPs) family, Tat peptide has been shown to deliver proteins, macromolecules, nanoparticles, and liposomes of sizes 2 nm to 200 nm across the cell membrane both in cell culture and *in vivo* applications, making them very attractive for various intracellular delivery applications. To validate the intracellular delivery of mMIONs, we used two reporting modalities: fluorescence imaging and magnetic contrast. To obtain fluorescence imaging capability, the mMIONs were conjugated with TxRed dye. We found that the iron oxide nanoparticles provided the magnetic contrast, as described below.

To further validate the delivery of the mMIONs into live MDBK cells, we measured T_2 (transverse) relaxation times and obtained MRI contrast images. These measurements are based on the induced changes in water relaxation caused by magnetic nanoparticles. We found that the T_2 relaxation time determined for cells with mMIONs was 604 ± 37 ms. In contrast, for control cells without mMIONS, the T_2 time was 1473 ± 75 ms. Therefore, in the mMION-treated cells, the T_2 time is at least a factor of two shorter than control cells, indicating significant internalization of magnetic nanoparticles. We further performed MR imaging using a 3T Siemens TRIO MRI system, and a shortened T_2 time resulted in a darker image. We found that when the same cell sample as used for T_2 measurements was imaged using an MRI system, cells containing incubated with mMIONs gave a darker image than control cells, once again indicating the presence of the relaxation agent inside cells.

D. Demonstration of the Contrast Mechanism by Clustering

To demonstrate the clustering-induced contrast in MRI, oligonucleotide probes of varying lengths are used to cluster two mMIONs together. Hybridization to the target oligonucleotide stiffens the connection, inducing a structural change and thereby

increasing the separation distance between the two MIONs. The detectable signal is a change in the water relaxation characteristics of the magnetic probe. T_2 measurements were made of mMIONs with oligonucleotides of different lengths and conformations in the absence and presence of their complementary strands. Upon hybridization, all preparations showed a decrease in R2, suggesting that the unhybridized structure held the mMIONs in closer proximity to each other than the more extended hybridized structure. The change in R2 upon hybridization differed depending on the configuration being studied.

Upon hybridization to a complementary target, a 40-nucleotide hairpin (sequence = 5'-thiol AAA AAG CAG CCA GCC AGT CTA GTC TAG TTT GCT GCA AAA A thiol-3') with mMIONs attached at both the 5' and 3' end changed from the closed conformation to an open conformation, increasing the distance between the two magnetic particles and decreasing their R2 (mM-1*s-1) by 30%. When a probe consisting of a random coil oligonucleotide between two magnetic nanoparticles hybridized to its complementary target, the two attached MIONs moved from close proximity to a more extended structure. We found that, as a result of this structural transition, R2 decreased. This change in R2 varied with the length of the oligonucleotide. Specifically, when the length of the polyA oligonucleotide holding the two MIONs together increased (from 10 adenosines to 40 adenosines), the change in R2 (R2 of hybridized duplex minus that of random coil) decreased. Computer simulations confirmed trends observed experimentally, i.e., the distance between MIONs contributes significantly to observed R2 changes upon hybridization. These simulations also show that the most significant changes in R2 occur at short (<20 nm) distances between MIONs.

E. Magnetic Nanoparticle as an MRI Contrast Agent

In conclusion, we found that the coating of small, uniformly-sized iron oxide nanoparticles resulted in an MRI contrast agent with a narrow size distribution that is biocompatible, water soluble, stable, and has a functionalized surface to which multiple ligands can be attached. Using the Tat peptide, we found very rapid (within 1 hour) delivery of mMIONs into living cells, which was validated using both fluorescence imaging and MRI contrast measurement. We believe that the surface of these mMIONS provides a flexible platform to which proteins, nucleic acid probes and a variety of ligands for cell delivery and targeting can be attached. In particular, separation of the reverse-micelle based synthesis and PEG-based coating steps allowed for the generation of magnetic nanoparticles with very uniform size (10% variation in diameter). Because of their small sizes (10-15 nm), these functionalized mMIONs are well suited as a contrast agent for probing intracellular events with less perturbation than other currently used iron oxide-based molecular probes. The small and uniform size may also confer an advantage in clustering-based molecular switching techniques for specific detection of gene expression in deep tissue using MRI. Therefore, the development of functionalized mMIONs in this study is a crucial step towards developing novel intracellular molecular probes for MRI-based deep tissue imaging.

F. The Development of a New MRI Contrast Method

We have developed a new MRI contrast method based on saturation by off-resonance irradiation. We found that by calculating the off-resonance saturation ratio, $1-M_{sat}/M_o$, where M_{sat} is the image taken with saturation pulses, much higher signal-to-

noise ratio can be obtained. This was demonstrated by performing FLASH imaging of mMION in aqueous solution, with iron concentrations of 0.0025, 0.01, 0.02, 0.03, 0.06, 0.15 and 0.45 mM, as well as in Agar phantom (2%) solution with iron concentrations of 0.5, 0.25, 0.125, 0.063 and 0.031 mM. The reason to use Agar is that Agar displays an effect called magnetization transfer which also results in a signal decrease when an off-resonance pulse is applied. This effect appears in tissues throughout the body and Agar is often used in phantoms to represent these tissues. We have clearly demonstrated that with the saturation by off-resonance method, small changes in mMION concentration can be images using MRI.

4. PERSONNEL SUPPORTED

Three senior investigators, two postdoctoral fellows, one technician, and four PhD students have been supported by the DARPA-AFOSR grant. They are:

Dr. Gang Bao, Professor, Principal Investigator, Georgia Institute of Technology

Dr. Shuming Nie, Associate Professor, Co-Principal Investigator, Emory University

Dr. Xiaoping Hu, Professor, Co-Principal Investigator, Emory University

Dr. Phil Santangelo, Postdoctoral Fellow, Georgia Institute of Technology

Dr. Leslie LaConte, Postdoctoral Fellow, Georgia Institute of Technology

Brent Cox, Technician, Georgia Institute of Technology

Nitin Nitin, Ph.D. student, Georgia Institute of Technology

Omar Zurkiya, M.D./Ph.D. student, Emory University

Tushar Sathe, Ph.D. student, Emory University

Daniela Caruntu, Ph.D. student, University of New Orleans

5. PUBLICATIONS

G. Bao, A. Tsourkas and P. J. Santangelo, "Engineering Nanostructured Probes for Sensitive Intracellular Gene Detection". *Mechanics and Chemistry of Biosystems*, 1, 23-36 (2004).

P.J. Santangelo, B. Nix, A. Tsourkas and G. Bao. "Dual FRET molecular beacons for mRNA detection in living cells". *Nucleic Acids Res.*, 32, e57 (2004).

N. Nitin, P. J. Santangelo, G. Kim, S. Nie and G. Bao. "Peptide-linked molecular beacons for efficient delivery and rapid mRNA detection in living cells", *Nucleic Acids Res.*, 32, e58 (2004).

N. Nitin, L. E. W. LaConte, O. Zurkiya, X. Hu and G. Bao, "Functionalization and peptide-based delivery of magnetic nanoparticles as an intracellular MRI contrast agent", *J. Biological Inorganic Chemistry*, 9, 706-712 (2004).

L. E. W. LaConte, N. Nitin and G. Bao, "Magnetic nanoparticle probes", *Nanotoday*, May 2005 issue, 32-38 (2005).

6. INTERACTIONS/TRANSITIONS

Three presentations were given by the PI which reflected the work supported by the DARPA-AFOSR grant:

1) Invited lecture for the Masterclass, UC San Diego, Department of Bioengineering, San Diego, CA. May 2003: 'Molecular Biomechanics: Opportunities, Approaches and Challenges'.

2) ASME 2003 Summer Bioengineering Conference, Key Biscayne, Florida, June 2003. 'High-Efficiency peptide-based delivery of proteins, nucleic acids and molecular probes into living cells'

3) Invited talk, Special Symposium on the Mechanical and Materials, University of Wyoming, Laramie, August, 2003. 'Playing with biomoleculars'.

4) MIT Single Cell Mechanics Conference, Cambridge, MA, October 2004. "Molecular imaging of single cell dynamics", invited talk.

5) 228 American Chemical Society Meeting, Philadelphia, PA, August 2004. "Sensitive mRNA detection in single living cells", invited talk.

6) ICCES'04 Madeira, Portugal, July 2004. Keynote Lecture. "Nanostructured Molecular Probes for Gene Detection in Living Cells".

7) Swiss Federal Institute of Technology, ETH Hönggerberg, Zürich, Switzerland, Jan. 2005. "Nanostructured probes for specific gene detection in living cells", invited seminar.

8) University of Pittsburgh, Department of Mechanical Engineering, Pittsburgh, PA, April 2005 "Nanostructured Probes for In Vivo Molecular Imaging", invited seminar.

9) University of Illinois at Urbana-Champaign, Department of Mechanical and Industrial Engineering, April 2005, "Engineering Nanostructured Probes for Living Cell Gene Detection", invited seminar.

10) Harvard University, Division of Engineering and Applied Sciences, May 2005. "Engineering nanoprobes for imaging gene expression dynamics", invited seminar.

7. INVEMNTIONS AND PATENT DISCLOSURES

We filed a full patent application as a result of the DARPA-AFOSR grant: *Multifunctional Magnetic Nanoparticle Probes for Molecular Imaging*, filed in the USPTO October 25, 2003, with co-inventors Gang Bao, Nitin Nitin and Shuming Nie.

8. HONORS AND AWARDS

1) **Gang Bao**, 2005 Outstanding Achievement in Research Program Development Award, Georgia Institute of Technology
2) **Gang Bao**, 2005 Sigma Xi Best Paper Award, Georgia Tech Sigma Xi Chapter

REPORT DOCUMENTATION PAGE

AFRL-SR-AR-TR-05-

0226

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	8 Jun 05	FINAL - 01 May 03 to 31 Oct 04

4. TITLE AND SUBTITLE
Multifunctional Magnetic Nanoparticle Probes for Deep-Tissue Imaging

5. FUNDING NUMBERS
F49620-03-1-0320

6. AUTHOR(S)
Bao, Gang

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Biomedical Engineering
Georgia Institute of Technology adn Emory University
Atlanta, GA 30332

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
AFOSR/NL
875 North Randolph Street
Suite 325, Rm 3112
Arlington, VA 22203

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Approve for Public Release: Unlimited Distribution

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

During the Phase I studies of the project, we have functionalized iron-oxide magnetic nanoparticle surface with a phospholipid-PEG coating. The resulting nanoparticles are water-soluble and suitable for bioconjugation of oligonucleotides and peptides. We have conjgated linear and hairpin oligonucleotide probes to magnetic nanoparticles and confirmed hybridization-induced clustering. We have developed a peptide-based delivery method for nanoparticle probes. This method has very high delivery efficiency and fast kinetics compared with other transfection-based methods. We have performed spectroscopic and MR imaging studies of the effect of magnetic nanoparticle probe coating and correlated the experimental measurements with theoretical prediction. We have aslo developed a saturation by off-resonance irradiation method which gives better signal-to-noise ratios. Taken together, these studies have greatly advanced the development of magnetic nanoparticle probes as MRI contrast agent for deep tissue imaging.

14. SUBJECT TERMS			15. NUMBER OF PAGES: 7
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

6-15-05